STUDIES ON THE ACTIVITY OF THE STANDARDIZED EXTRACTS OF *PIPER BETLE* L. AND *PIPER NIGRUM* L. ON *MYCOBACTERIUM TUBERCULOSIS*: CELLULAR AND METABOLOMICS APPROACHES

By

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Thesis submitted in fulfillment of the requirements for the degree of Master of Pharmaceutical Sciences

2012

ACKNOWLEDGEMENTS

First of all, I want to take this opportunity to thank Allah s.w.t. because His supremacy has given me many ideas to accomplish this thesis. I would also like to express my gratitude to my mother Fatimah binti Abu Samad and my father Untung bin Ahmad Idris. I always pray "Ya Allah, please give my parents the most beautiful place in heaven".

I would like to thank my supervisor Assoc. Dr. Hjh. Pazilah Ibrahim and my co-supervisor Prof. Zhari Ismail for their invaluable advice and contributions to this work. May Allah reward you for all your kindness and help.

I would also like to extend my thanks to my labmates especially Ang, Adila, Anis, Biha, Naveen and Thaiga for making the lab an enjoyable working environment and giving me support and advices. I am also indebted to Pn. Salida, En. Razak Hamdan, En. Syamsuddin and all staffs of School of Pharmaceutical Sciences, USM for their technical assistance and kind knowledge-sharing. Thanks also to Microscopy Unit of School of Biological Sciences, USM and Makmal Veterinary Bukit Tengah for their willingness to give facilities to my work. My thanks also go to the CAMO Software Company for the provision and permission to use their product The Unsrambler X version 10.1. I am grateful to Research University Grant for their financial support to my work and also to Ministry of Higher Education of Indonesian Republic (DIKTI) who have sponsored my study.

Finally, to my beloved wife, Rismalina and beloved daughter, Aisyah Halwa Mawaddah, my deepest love for their prayers, understanding, patience and loyalty. Not forgetting, my gratitude to my family for their moral support and encouragement. Thank you again.

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LIST OF ABBREVIATIONS

µg/ml	:	microgram per millilitre
µl	:	microlitre
^{O}C	:	degree celsius
1D-NMR	:	one dimensional nuclear magnetic resonance spectroscopy
2D-NMR	:	two dimensional nuclear magnetic resonance spectroscopy
A	:	adenine
ADC	:	albumin dextrose catalase
AIDS	:	acquire immunodeficiency syndrome
As	:	arsenic
ATCC	:	American type culture collection
ATP	:	adenosine triphosphate
ATPase	:	adenosine triphosphatase
ATR	:	attenuated total reflectance
ATR-FT-IR	:	attenuated total reflectance-fourier transform infrared spectroscopy
BCG	:	Bacille Calmette Guerin
BHC	:	benzene hexachloride
BLE	:	ethanol-water extract of *Piper betle* leaf
BLF	:	ethyl acetate fraction of *Piper betle* leaf
BST	:	brine shrimp test
C	:	cytosine
Cd	:	cadmium
CE	:	capillary electrophoresis

CFU	:	colony forming unit
CFU/ml	:	colony forming unit per millilitre
$CHCl_3$	:	chloroform
CHL	:	Chinese hamster lung
CLL	:	chronic lymphocytic leukaemia
cm	:	centimetre
CO_2	:	carbon dioxide
Cu	:	cuprum (copper)
DDT	:	dichlorophenyltrichloroethane
DIMS	:	direct infusion mass spectroscopy
DMSO	:	dimethylsulfoxide
DNA	:	deoxyribonucleic acid
DOT	:	directly observed treatment
DOTS	:	directly observed treatment short-course
EH	:	isoniazid - ethambutol
EPA	:	environment protection agency
EtOAc	:	ethyl acetate
EtOH	:	ethanol
EtOH /H_2O	:	mixture solution of ethanol and water
FDC	:	fixed-dose combination
Fe	:	ferrum (iron)
FIA-ICR-MS	:	flow injection analysis - ion cyclotron resonance - mass spectroscopy
FT-IR	:	fourier transform infrared spectroscopy
FTIR-ICR-MS	:	fourier transform infrared spectroscopy - ion cyclotron resonance - mass spectroscopy

G	:	guanine
g	:	gram
GAP	:	good agricultural practice
GC	:	gas chromatography
GC/GC	:	gas chromatography/gas chromatography
GC/GC-TOF-MS	:	gas chromatography/gas chromatography – time of flight mass spectroscopy
GC-MS	:	gas chromatography – mass spectroscopy
GI	:	growth index
GMP	:	good manufacturing practice
h	:	hour(s)
H_2O	:	dihydrogen monoxide or water
HCA	:	hierarchical cluster analysis
HeLa	:	human cervical carcinoma
Hg	:	hydragyrum (mercuri)
HIV	:	human immunodeficiency virus
HMDS	:	hexamethyldisilazane
HPLC	:	high performance liquid chromatography
HPLC-DAD	:	high performance liquid chromatography with diode-array detection
HPLC-MS/MS	:	high performance liquid chromatography- mass spectroscopy/ mass spectroscopy
HPTLC	:	hight performance thin layer chromatography
IR	:	infrared spectroscopy
KatG	:	catalase peroxidase of *Mycobacterium tuberculosis*
kg	:	kilogram
L	:	leaf

LAM	:	lipoarabinomannan
LC_{50}	:	lethal dose concentration that kills 50 percent of brine shrimp
LC-MS	:	liquid chromatography - mass spectroscopy
LLS	:	local least squares
LOD	:	limit of detection
LOQ	:	limit of quantitation
M	:	molar concentration
Md	:	moderate toxic
MDR-TB	:	multidrug resistant tuberculosis
mg	:	milligram
MIC	:	minimum inhibitory concentration
ml	:	millilitre
ml/min	:	millilitre per minute
mm	:	millimeter
Mn	:	manganese
MS	:	mass spectroscopy
MTB	:	*Mycobacterium tuberculosis*
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	:	nicotinamide adenine dinucleotide plus hydrogen
NADPH	:	nicotinamide adenine dinucleotide phosphate plus hydrogen
NLE	:	ethanol extract of *Piper nigrum* leaf
NLF	:	ethyl acetate fraction of *Piper nigrum* leaf
nm	:	nanometer
NMR	:	nuclear magnetic resonance spectroscopy
NO_2^-	:	nitrite

NO_3^-	:	nitrate
NRA	:	neutral red assay
NT	:	non toxic
OADC	:	oleic acid albumin dextrose catalase
Pb	:	plumbum (lead)
PBS	:	phosphate buffer saline
PC	:	principal component
PCA	:	principal component analysis
pH	:	the power of hydrogen
PLS	:	partial least squares
ppm	:	part per million
Redox	:	reduction - oxidation
Rf	:	retention factor
RH	:	rifampicin - isoniazid
RHZ	:	rifampicin - isoniazid - pyrazinamide
RHZE	:	rifampicin – isoniazid – pyrazinamide - ethambutol
RNA	:	ribonucleic acid
RSD	:	relative standard deviation
S	:	stem
St	:	strong toxic
SD	:	standard deviation
SEM	:	scanning electron microscopy
STOCSY	:	statistical total correlation spectroscopy
T	:	thymine
TACO	:	tryptopan-aspartate containing coat protein

TB	:	tuberculosis
TEM	:	transmission electron microscopy
TEMA	:	tetrazolium microplate assay
TLC	:	thin layer chromatography
UK	:	the United Kingdom
UNCT	:	the United Nation Country Team
USA	:	the United States of America
v/v	:	volume per volume
W	:	weakly toxic
WHO	:	World Health Organization
w/v	:	weight per volume
XDR-TB	:	extensively drug resistant tuberculosis
Zn	:	zinc

KAJIAN EKSTRAK *PIPER BETLE* L. DAN *PIPER NIGRUM* L. TERPIAWAI TERHADAP *MYCOBACTERIUM TUBERCULOSIS*: PENDEKATAN SELULAR DAN METABOLOMIK

ABSTRAK

Peningkatan kes-kes tuberkulosis (TB) yang rintang terhadap ubat-ubatan anti-TB telah mendorong kepada keperluan segera untuk meneroka ubat anti-TB yang baru yang sebaik-baiknya daripada tumbuhan ubatan tempatan. Walau bagaimanapun, pada dekad yang lepas tidak banyak perhatian telah diberikan ke atas aktiviti anti-TB tumbuhan ini dari aspek mikrobiologi dan metabolomik.

Kajian ini dijalankan untuk menguji aktiviti antimikobakteria terhadap ekstrak dan fraksi *Piper betle* and *Piper nigrum* terpiawai. Ekstrak dan fraksi tersebut masing-masing disediakan dengan kaedah maserasi dan partisi cecair. Aktivitinya diuji mengguna kaedah pencairan mikro tetrazolium. Kesan fraksi aktif terhadap pertumbuhan *Mycobacterium tuberculosis* juga dikaji di bawah pemerhatian mikroskop cahaya dan imbasan elektron. HPLC dan gabungan spektroskopi FT-IR dengan PCA diguna untuk memprofilkan ekstrak dan fraksi aktif. Ujian toksisiti ekstrak dan fraksi dijalankan mengguna kaedah Brine Shrimp. Keputusan menunjukkan bahawa ekstrak *P. betle* dan *P. nigrum* memiliki aktiviti antimikobakteria dengan julat MIC 100 µg/ml hingga 500 µg/ml manakala julat MIC fraksinya ialah 25-100 µg/ml. Penilaian di bawah mikroskop cahaya menunjukkan bahawa kedua-dua fraksi telah memberi kesan terhadap pertumbuhan sel *M. tuberculosis* menyebabkan hilangnya ciri ketahanan asid dan perubahan morfologi. Di bawah mikroskop imbasan elektron sel tersebut nampak keriput dan kosong kerana pelepasan bahan sitoplasma daripada sel pecah. Analisis HPLC menunjukkan bahawa ekstrak dan fraksi *P. betle* mengandungi eugenol masing-

masing 0.065% dan 0.010%. Manakala ekstrak dan fraksi *P. nigrum* masing-masing mengandungi piperin sebanyak 1.215% dan 0.060%. Analisa prinsip komponen (PCA) juga telah membezakan ekstrak etanol/air (1:1) daun *P. betle*, ekstrak etanol daun *P. nigrum*, dan fraksi etil asetat dari kedua-dua ekstrak berasaskan spektrum FT-IR. Ujian toksisiti menunjukkan bahawa ekstrak aktif mempunyai toksisiti rendah dengan LD_{50} di bawah 1000 µg/ml manakala LD_{50} fraksi etil asetat *P. betle* dan *P. nigrum* masing-masing adalah 90.78 µg/ml dan 137.90 µg/ml. Kajian ini menunjukkan bahawa kedua-dua tumbuhan *P. betle* dan *P. nigrum* memiliki potensi aktiviti antimikobakteria.

STUDIES ON THE ACTIVITY OF THE STANDARDIZED EXTRACTS OF *PIPER BETLE* L. AND *PIPER NIGRUM* L. ON *MYCOBACTERIUM TUBERCULOSIS*: CELLULAR AND METABOLOMICS APPROACHES

ABSTRACT

The rising of drug-resistance tuberculosis (TB) cases has prompted an urgent need for a new anti-TB drug preferably from local medicinal plants. However, in the last decade not much attention has been given to the microbiology and metabolomics aspects of these plants regarding to their anti-TB activity.

This study was carried out to test the antimycobacterial activity of standardized extracts and fractions of *Piper betle* and *Piper nigrum*. The extracts and fractions were prepared with maceration and liquid-liquid partition method, respectively. The activity was tested using tetrazolium microdilution assay. The effects of the active fraction on the growth of *Mycobacterium tuberculosis* was also studied under light and scanning electron microscopy observation. HPLC and FTIR spectroscopy in combination with PCA were used to profile the extract and fraction. Toxicity testing of the active extracts and fraction was performed using Brine Shrimp method. The results indicated that extracts of *P. betle* and *P. nigrum* exhibited antimycobacterial activity with MIC values ranging from 100 µg/ml to 500 µg/ml while the MIC of the fractions was 25-100 µg/ml. Evaluation under light microscopy showed that the active fractions affected the growth of mycobacterial cells causing loss of acid-fastness and morphological changes. Under scanning electron microscopy the affected cells appeared wrinkled and empty due to release of cytoplasmic materials from ruptured cells. HPLC analysis showed that the extract and fraction of *P. betle* contain 0.065% and 0.010% eugenol, respectively. Meanwhile extract and fraction of *P. nigrum* contain 1.215% and 0.060% piperine,

respectively. Principal component analysis (PCA) was able to differentiate the leaf ethanol/water (1:1) extract of *P. betle*, the leaf ethanol extract of *P. nigrum*, and its ethyl acetate fractions based on the FT-IR spectra. Toxicity test indicated that the active extract had low toxicity with LD_{50} >1000 µg/ml while the LD_{50} of the ethyl acetate fractions of *P. betle* and *P. nigrum* were 90.78 µg/ml and 137.90 µg/ml, respectively. This study indicated that both *P. betle* and *P. nigrum* have potential antimycobacterial activity.

CHAPTER 1
INTRODUCTION

1.1 Tuberculosis

Tuberculosis (TB) remains a serious problem faced by many countries in the world. TB is a major cause of illness and death worldwide, especially in Asia and Africa. Globally, 9.4 million new cases and about 1.7 million deaths from TB occurred in 2009. This was equal to 4,700 deaths per day or about 3 people die every minute due to TB. The number of new cases increased in the last decades as shown in Figure 1.1. Most cases were in South East Asia, Africa and Western Pacific regions with percentages 35%, 30% and 20% of global cases, respectively (World Health Organization [WHO], 2010a).

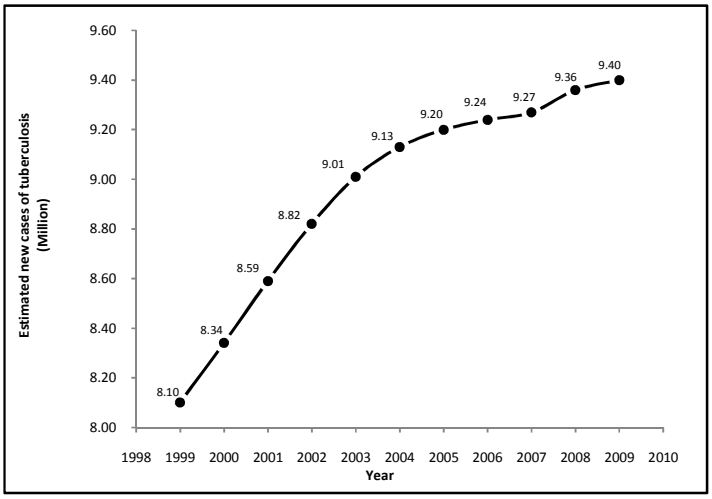

Figure 1.1 Estimated new cases of tuberculosis in the world during 1999-2009 (WHO, 2010a)

In Malaysia, tuberculosis remains a common disease. WHO has classified Malaysia as an intermediate TB burden country where 63 new cases per 100,000 people in 2009 were reported. However, it still remains a serious problem for the country (WHO, 2010b). The United Nation Country Team [UNCT] (2011) reported

the number of new cases increased from 10,873 in 1990 to 18,102 in 2009. The highest cases occurred in Sabah and Selangor with the number of cases 5,249 and 2,342, respectively. In addition, TB in Malaysia belongs in the top five main notifiable diseases which leads to mortality (WHO, 2010b).

The increasing incidence of tuberculosis globally is partly due to the HIV/AIDS pandemic. Persons with TB/HIV co-infected are more likely to develop active TB disease than persons without HIV. For example, the number of HIV-positive people with infected TB increased from 0.6 million in 2007 to 1.4 million in 2008 (WHO, 2009). TB is one cause of death in people with HIV. The death of HIV patients associated with TB was approximately 380,000 in 2009 (WHO, 2010c). In Africa, HIV is the most important factor contributing to the increase in the incidence of TB since 1990 (WHO, 2010d). Autopsy studies on HIV-infected adult who died in Africa showed that 30% to 40% was due to tuberculosis. In addition, TB killed about one in five of all deaths among HIV-infected children (Chaisson & Martinson, 2008). In Malaysia, HIV pandemic was also involved in the development of TB cases. This can be seen from the increasing of TB-HIV co-infection cases from 933 cases in 2002 to 1,644 in 2009 (UNCT, 2011).

The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* (MDR-TB) has also contributed to the expansion of the disease. According to WHO (2010e), MDR-TB is defined as a form of TB which is resistant to at least isoniazid and rifampicin. WHO estimated that 440,000 people had MDR-TB worldwide in 2008 and that a third of them died. Almost 50% of MDR-TB cases worldwide are estimated to occur in China and India. In Africa, estimates show 69,000 cases emerged, the vast majority of which went undiagnosed. WHO also reported that in Russia, one in four people with tuberculosis had MDR-TB form of the disease that

can no longer be treated with standard drugs regimens (WHO, 2010d). MDR-TB is also reported in Malaysia. In 2004, the number of MDR-TB cases were 13. The number has increased to 17 cases in 2005 and 42 cases in 2006 (Glaziou, 2007). Meanwhile, 31 new cases of MDR-TB were detected in 2008 and then increased to 55 cases in 2009 (WHO, 2010e).

In addition, the unpleasant side-effects of TB drugs and a relatively long course of treatment contributed to increasing the rate of noncompliance to treatment regimen which in turn led to treatment failure and the development of drug resistance (Bisth et al., 2006).

1.1.2 Genus *Mycobacterium*

Genus *Mycobacterium* belongs to suborder Corynebacterineae which is characterized by the presence of mycolic acid in the cells (Stackebrandt et al., 1997). The genus *Mycobacterium* comprises more than 100 species (Euzeby, 2011). Based on the rate of growth, genus *Mycobacterium* is classified into rapidly growing and slowly growing organisms. The rapid grower commonly forms colonies within 7 days of incubation. This include *Mycobacterium fortuitum*, *Mycobacterium peregrinum*, *Mycobacterium abscessus*, *Mycobacterium chelonae*, and *Mycobacterium thermoresistible*. Meanwhile, the slow growers requires longer incubation and this include *Mycobacterium kansasii*, *Mycobacterium marimum*, *Mycobacterium asiaticum*, *Mycobacterium scrofulaceum*, *Mycobacterium szulgai*, *Mycobacterium xenopy*, *Mycobacterium celatum*, *Mycobacterium gordonae*, *Mycobacterium flavescens*, *Mycobacterium avium*, *Mycobacterium terrae*, *Mycobacterium tuberculosis*, *Mycobacterium shimoidae*, and *Mycobacterium genavense* (Runyon, 1959). Both types of genus *Mycobacterium* are commonly

straight or curve rods that grow to be filaments in aerobic conditions. The cells can also form a branch during the growth process (Saviola & Bishai, 2006). The cells are then divided into two daughter cells by binary fission (Singh et al., 2010).

One of the genus *Mycobacterium* related with tuberculosis disease is *Mycobacterium tuberculosis*. In the Taxonomicon Project 2000 developed by Brands (2011), *M. tuberculosis* is classified into:

Super Kingdom : Bacteria/Monera

Phylum : Actinobacteria

Class : Actinobacteria

Subclass : Actinobacteridae

Order : Actinomycetales

Suborder : Corynebacterineae/Mycobacteria

Family : Mycobacteriaceae

Genus : *Mycobacterium*

Species : *Mycobacterium tuberculosis*

M. tuberculosis is the etiologic agent of tuberculosis in humans. The tubercle bacilli was found by Robert Koch in 1882 (Kanai, 1990). The discovery inspired scientists to find a remedy for tuberculosis. In 1921, Calmette and Guerin successfully isolated a live strain of *Mycobacterium bovin* from a cow infected with tuberculosis. The strain was subcultured and tested on cows and guinea pigs for thirteen years and then found to be less virulent for the animals. The attenuated strain was known as Bacille Calmette Guerin (BCG) vaccine which was used to protect humans against tuberculosis (WHO, 2011a). In 1944 streptomycin was discovered by Waksmann as the first antituberculosis drug. Since then, a number of antituberculosis

drugs were discovered, including para-aminosalicylic, isoniazid, pyrazinamid and cycloserine, ethionamide, rifampicin and ethambutol (Robson & Sullivan, 1963).

M. tuberculosis is a straight or slightly curved rod with length ranging from about 1 - 4 µm and 0.3 - 0.6 µm thick, occuring singly or in a form of strands (Youmans, 1979). Although *M. tuberculosis* belongs to gram-positive bacteria, they are difficult to stain with the Gram stain method. However, carbol fucshin or Ziehl-Neelsen method is used to stain mycobacterial cells. The cells appear small red or pink rod under microscopic examination (Grosset et al., 2000). The bacilli grow slowly with the generation time *in vitro* being 14 - 15 hours. Colonies appear in about two weeks and may sometimes take up to eight weeks. The bacilli grow at an optimum temperature of 37^0C and at pH 6.4 - 7.0. However, growth does not occur below 25^0C or above 40^0C (Youmans, 1979). The growth of bacilli can be seen by morphological changes of the cells. The rod cells grow to be elongated cells after 24 hours incubation. The cells then divide to generate new mature cells. The cells also branched during the dividing process to form V-shaped cells (Dahl, 2004).

M. tuberculosis is an obligate aerobe and grows luxuriantly in both liquid and solid media. The additional 0.5% glycerol as well as 0.2% sodium pyruvate improves the growth of *M. tuberculosis* (Grosset et al., 2000). The excellent media of Middlebrook's 7H9, 7H10 and 7H11 for growth of the bacilli has been reported. On solid media, *M. tuberculosis* forms dry, rough, raised, and irregular colonies with a wrinkled surface. They are creamy white, becoming yellowish or buff coloured on further incubation. In liquid media without dispersing agent such as polyoxyethylene sorbitan monooleate (tween 80) the growth begins at the bottom, creeps up the sides and forms a prominent surface pellicle which may extend along the sides above the medium (Youmans, 1979).

The growth cycle of *M. tuberculosis* has four major phases (Figure 1.2). Typical phases of mycobacteria can be demonstrated by inoculating the cells in fresh medium under favorable conditions and the cells are counted at frequent intervals (Levinson, 2006).

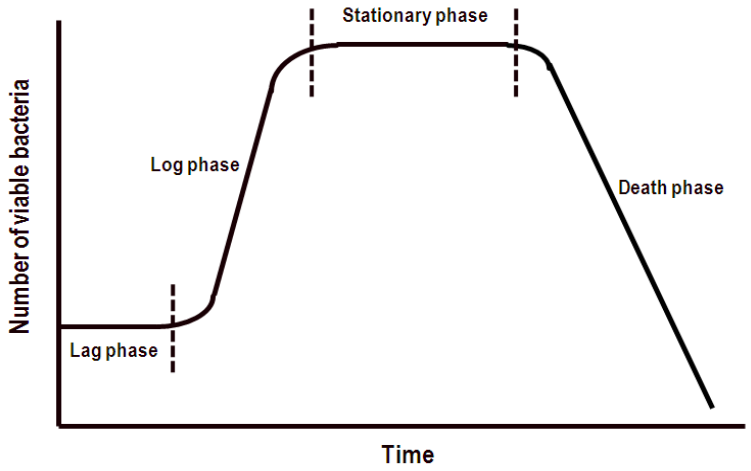

Figure 1.2 Growth cycle of *Mycobacterium tuberculosis* (Levinson, 2006)

The first is the lag phase where by the cells adapt to new environment and the cell population remains unchanged. In this phase, the cells grow in size and mass, store nutrients, synthesize enzymes, and prepare for cell division. Subsequent phase of the growth is the log phase. During this phase, the cells divide at a constant rate. The cells also uptake and metabolize nutrients at maxiumum speed. However, when availability of nutrients become limited, the growth of the cells slows down. In the stationary phase, the multiplication rate of the cells decreases due to exhaustion of nutrients in the culture medium. Thus, the number of viable cells remains stationary. The final phase of the mycobacterial growth is the death phase. In this phase, the cell division completely ceases due to exhaustion of nutrients and accumulation of toxic products. In addition, the presence of autolytic enzymes also causes cell death (Todar, 2008).

1.1.3 Pathogenicity and Transmission of *Mycobacterium tuberculosis*

Pathogenicity is defined as the ability of the organism to produce disease in a host. *M. tuberculosis* has the ability to invade and multiply within macrophages of the host. The pathogenicity of *M. tuberculosis* often relates to the effort of the mycobacteria to survive in the macrophage condition. Presence of tubercle bacilli will stimulate the immune response of the host. The macrophages produce lysosome hydrolase enzyme through the fusion with lysosomes (Smith et al., 2007). In addition, the macrophages also create low pH condition. The presence of the enzyme and low pH induces killing and digestion of the mycobacteria (Jordao et al., 2008).

In addition, *M. tuberculosis* also has a cell envelope which contains a cell membrane and a peptidoglycan layer. The cells also have a thick hydrophobic layer of mycolic acids esterified to the cell wall called the mycolyl arabinogalactan. The unique composition of mycobacterial cell envelope is associated with its pathogenicity (Glickman & Jacobs, 2001). Lipoarabinomannan (LAM) is a major constituent of mycobacterial cell wall. The LAM has the ability to inhibit fusion of phagosome with lysosome. Consequently, the killing mechanism of phagosome is prevented (Pieters, 2008). LAM can also inactivate the macrophages through inhibition of interferon-γ. Interferon-γ is an activating agent of the macrophage to generate reactive oxygen and nitrogen which damage the mycobacteria (Meena & Rajni, 2010). However, the mycobacteria has the ability to develop multiple strategies for survival. They inactivate the reactive oxygen via formation of a catalase-peroxidase enzyme called KatG. The KatG is an enzyme that catalizes the decomposition of hydrogen peroxide to water and oxygen. In addition, mycobacteria will activate the proteamose function in nitric oxide stress. Proteamose is a enzyme that serves to eliminate protein damaged by the reactive nitrogen. Furthermore, the

protein will be excreted out of the cells (Pieters, 2008). On the other hand, mycobacteria can inhibit the acidification of macrophage through activation of the proton ATPase-dependent to stabilize the environment. In addition, mycobacteria can also recruitment and inactivate of tryptophan-aspartate containing coat protein (TACO) on phagosome. In the process of killing bacteria, TACO is responsible to deliver the bacterial toward lysosomes (cellular organelles that contain acid hydrolase enzymes to break down waste materials). Consequently, inactivation of TACO will avoid the mycobacteria to the destruction of lysosomes (Meena & Rajni, 2010).

The ability of *M. tuberculosis* to survive and remain dormant in the host cells for years provides potential to be activated when the immune system becomes inadequate. TB is a communicable disease which spreads via aerosols. During coughing, sneezing, talking or spitting, people infected with TB produced droplet nuclei containing *M. tuberculosis* into the air (Varaine et al., 2010; Youmans, 1979). Infection occurs when a susceptible person inhales the droplet nuclei. The droplet enters the body through the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli. The person will become sick within 2 – 12 weeks after infection depending on the immune response (Jensen et al., 2005).

1.1.4 Multidrug Resistant Tuberculosis

Multidrug resistant tuberculosis (MDR-TB) is defined as resistant to at least isoniazid and rifampicin (Spardling & Ridzon, 2004). Two broad categories of MDR-TB are recognized, that is primary and acquired. Primary resistance refers to drug resistance of the bacilli from a patient who has never received treatment. Acquired or secondary resistance refers to resistance of the bacilli after exposure to

an antimicrobial agent, whereby the mutant bacilli have been selected for survival (Goble, 2000). The acquired resistance usually occurs as a result of non-adherence to TB drug regiments or treatment failure (Maurya et al., 2011). The resistance is associated with the use of a single TB drug to treat TB which is not enough to kill tubercle bacilli. In addition, poor compliance due to several reasons such as alcoholism, homelessness, drug addiction, and side effects of TB drugs also contribute to the acquired resistance. Other contributing factors include inadequacy of TB treatment management and quality of the antituberculosis drug (Jain & Dixit, 2008).

The other type of resistance is extensively drug resistant tuberculosis (XDR-TB). According to WHO (2010f), the XDR-TB is a form of tuberculosis caused by *M. tuberculosis* that is resistant to at least isoniazid and rifampicin plus any fluoroquinolone and any injectable second-line anti TB drug such as amikacin, kanamycin, and capreomycin. XDR-TB commonly occurs because of misuse and mismanagement of the second-line TB drugs. This may be contributed by several factors such as patients do not complete their second line regimens or due to inappropriate dose regiment and poor quality of drugs given by the health-care providers (WHO, 2007a).

WHO estimates the emergence of 25,000 cases of XDR-TB every year. There are 27 high XDR-TB burden countries such as Argentina, Canada, United States, France, Germany, Armenia, Russia Federation, China, India, Israel, South Africa, Thailand, Myanmar, and Australia. Meanwhile in Malaysia, as yet, there is no report of XDR-TB cases (WHO, 2011b).

The emergence of resistance to anti-tuberculosis drugs is recognized as a phenomenon that cannot be avoided. The resistance not only poses a public health

threat to successful control of TB but it also complicates the approaches to treatment of patients (Heifets & Cangelosi, 2009). The use or misuse of anti-tuberculosis drugs over the years has led to increasing prevalence of drug resistance, thus development of new effective drugs has become urgent (Cox et al., 2003; Nachega & Chaisson, 2003). In addition, the use of antituberculosis drug for MDR-TB and XDR-TB need longer duration treatment (2 years) which is costly and toxic (WHO, 2008a).

1.1.5 Treatment of Tuberculosis

Treatment of tuberculosis with effective chemotherapy can reduce the population of viable bacilli and consequently reduce the risk of transmission. Five drugs currently used as essential in the management of tuberculosis, which are also known as first line TB-drugs are isoniazid, rifampicin, pyrazinamid, streptomycin, and ethambutol. Other drugs such as para-aminosalicylic acid, kanamycin, cycloserin, capreomycin, viomycin, and ethionamide are used to treat patients with multiple drug resistant disease. These drugs which are known as second line TB-drugs are more expensive and more toxic than the first line (WHO, 1991).

The WHO-recommended strategy for tuberculosis control is directly observed treatment short-course (DOTS). The DOTS strategy is a short course chemotherapy based on appropriate diagnosis of TB with a minimum of six months. Chemotherapy for TB uses standardized drug regimens of fixed-dose combination (FDC) for all patients (WHO, 2002a). Treatment regimens for TB using FDC depend on categories of TB cases. The recommended treatment of first-line anti TB drug are presented in Tables 1.1 and Table 1.2 (WHO, 2002b).

Table 1.1 Recommended treatment regimens for tuberculosis adult patients

Patient body weight (kg)	**Initial phase**			**Continuation phase**		
	2 months			***4 months***		***or 6 months****
	Daily	or Daily	or 3 times per week	Daily	or 3 times per week	Daily
	RHZE (150+75+400+275) mg	RHZ (150+75+400) mg	RHZ (150+150+500) mg	RH (150+75) mg	RH (150+150) mg	EH (400+150) mg
30-39	2**	2	2	2	2	1.5
40-54	3	3	3	3	3	2
55-70	4	4	4	4	4	3
71 and more	5	5	5	5	5	3

R: rifampicin; H: isoniazid; Z: pyrazinamide; E: ethambutol

* 4RH may be replaced by 6 EH daily when supervision of treatment is not possible. However, preliminary data from a recent clinical trial have shown that 6EH is much less effective than 4RH in terms of cure, with higher failure and relapse rates.

** Number of drug FDC tablets.

Table 1.2 Recommended treatment regimens for tuberculosis children patients

Patient body weight (kg)	**Initial phase**	**Continuation phase**	
	2 months	***4 months***	
	Daily	Daily	or 3 times per week
	RHZ (60+30+150) mg	RH (60+30) mg	RH (60+60) mg
<7	1*	1	1
8-9	1.5	1.5	1.5
10-14	2	2	2
15-19	3	3	3
20-24	4	4	4
25-29	5	5	5

R: rifampicin; H: isoniazid; Z: pyrazinamide; E: ethambutol.

* Number of drug FDC tablets.

Directly observed treatment (DOT) means the patient swallows the FDC tablets in front of a trained health worker. This proposes to ensure the TB patient takes the right drugs, in the right doses, at the right intervals. Treatment of TB is carried out in two phases namely initial and continuation phase. In the initial phase, the drug regimen for new TB adult patient consisted of rifampicin, isoniazid, pyrazinamide and ethambutol (RHZE). However for children, only 3 drugs combination without ethambutol (RHZ) is given. In relapse case or treatment failure, ethambutol of RHZE combination is usually replaced with streptomycin. The duration of the initial phase is 2 months. In the continuation phase, all TB patients receive rifampicin and isoniazid combination for 4 months. Rifampicin usually

replaces ethambutol when there is a hypersensitivity reaction. In this case, the treatment duration is 6 months (WHO, 2002b).

The main problem related to FDC regimen is non-compliance of TB patients. This is because the regimen needs long duration periods to complete the TB treatment (Du Toit et al., 2006). Besides, the patient has to consume a large number of tablets every day and this may create frustration among the patients (Panchagnula et al., 2003). In addition, most anti-TB drugs can cause liver damage and renal failure. Once the patient experiences allergic or side-effects to any of drugs, the FDC regimen must be stopped or replaced with other anti-TB drugs (Bhowmik et al., 2008).

Although the chemotherapy is effective for treatment of tuberculosis but development of drug resistance and tolerance of the anti-TB drug used remain serious problem. The resistance is contributed by several factors such as poor compliance and non-adherence to drug regiments as well as failure of drug prescription and therapy monitoring (Rastogy & Falkinham, 1996; Schaberg, 1995; Horne, 1994). Hepatotoxicity, a side effect of anti-TB drugs particularly INH is a major factor in tolerance of the tuberculosis therapy. INH is most widely associated with incidence of hepatotoxicity during patient receiving chemotherapy. Therefore, new drugs without serious adverse effects are urgently needed to control tuberculosis disease (Schaberg, 1995). In addition, there has been no new anti-TB drugs introduced since ethambutol was discovered in 1957. Thus, there is an urgent need to search and develop new effective anti-TB drugs (Gautam et al., 2007; Tomioka & Namba, 2006).

1.2 Susceptibility Testing

Susceptibility testing is defined as measurement of the ability of an antimicrobial agent to kill or inhibit the growth of bacteria *in vitro* (Vandepitte et al., 2003). Bell et al. (2009) stated that the susceptibility of drug is usually made on the basis of breakpoints which is known as minimum inhibitory concentration (MIC). Andrews (2001) and Smaill (2000) defined the MIC as the lowest concentration of a drug that inhibits visible bacterial growth. Several susceptibility testing methods to determine the MIC value of antimycobaterial activity of natural products were introduced, namely: broth dilution, proportional, disk diffusion, radiometric, and colorimetric method.

1.2.1 Broth Dilution

Macrobroth or tube-dilution method is carried out by preparing two fold serial dilution of test sample in test tubes containing liquid medium. A bacterial suspension of 1-5 x 10^5 CFU/ml is added in these tubes. MIC determined after incubation is based on turbidity of visible bacterial growth (Jorgensen & Ferraro, 2009). The turbidity is recorded by measuring the optical density at 405 nm and growth curve plots for all concentration of the sample (Figure 1.2). The MIC is defined as the lowest concentration of the sample with no visible or detectable mycobacteria growth (Cos et al., 2006).

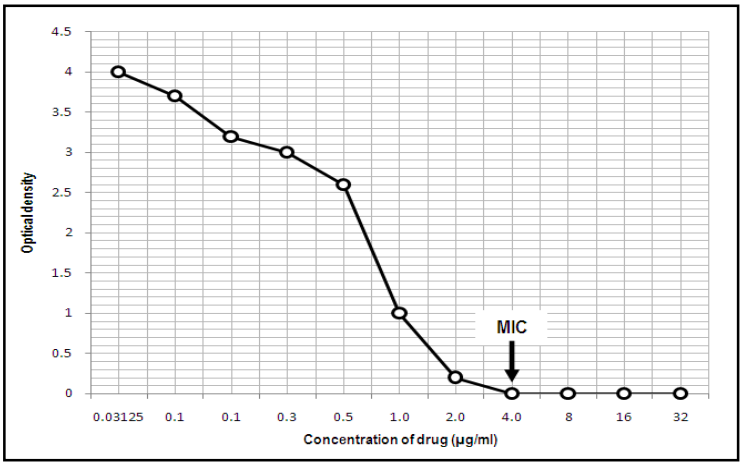

Figure 1.3 Growth curve with plot optical density vs concentration of drug (Cos et al., 2006)

The advantages of this method are that it is easy to perform, cheap, rapid, and the results are reproducible. However, the preparation of the drug solutions is tedious and could lead to error. In addition, this method requires the use of large amount of reagents (Jorgensen & Ferraro, 2009).

1.2.2 Proportional Method

This method is carried out by preparing different concentrations of test sample in agar plate. An inoculum of mycobacteria is transferred to the plate. After incubation, viable colony of the bacterial growth is counted for each concentration of test compound (Mendoza, 1998). MIC is defined as the lowest concentration of the sample which caused a 10^{-2} reduction in the viable counts compared to the respective control (Klingeren et al., 2007). The advantage of this method is that it can be used for testing of several mycobacterial isolates simultaneously. However, this method is time consuming (Mendoza, 1998).

1.2.3 Disk Diffusion

Disk diffusion method is prepared by placing a paper disc containing certain concentration of test sample on agar plate surface previously inoculated with mycobacteria. After incubation, mean diameters of growth inhibiton zone are recorded. Interpretation of zone sizes and the MIC is determined using Kirby–Bauer method as describe in Figure 1.3 (Vandepitte et al., 2003). The advantage of this method is that it does not require any special equipment and the results are easy to interpret. However, this method remains manually performed (Jorgensen & Ferraro, 2009).

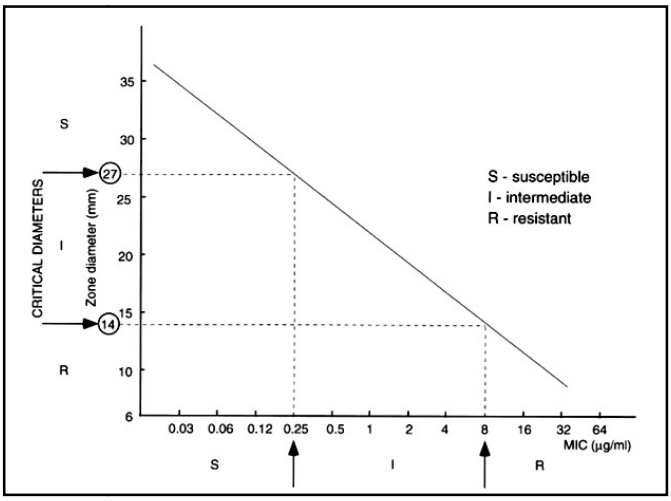

Figure 1.4 Interpretation of zone sizes by their relationship to the MIC (Vandepitte et al., 2003)

1.2.4 Radiometric

Radiometric is a method to detect the presence of mycobacteria based on their metabolism. This method is performed by preparing serial dilution of test sample in liquid medium containing ^{14}C-palmitic acid. The mycobacteria will metabolize the ^{14}C to carbon dioxide ($^{14}CO_2$) which is measured by BACTEC 460 instrument and reported as growth index (GI). This method can produce accurate

results in around 5 days (Ramachandran & Paramasivan, 2003). The test is interpreted on the day the GI in inoculum control diluted 1:100 reaches 20 or more. MIC is defined as the lowest concentration of the sample that inhibited more than 99% of the mycobacterial population. It was obtained as the lowest concentration in which the daily GI increases were less than the control and the final GI of the sample was not greater than 50 at the last day of experiment (Siddiqi et al., 1993). The main disadvantage is that this method is more expensive than conventional methods (Newton et al., 2000).

1.2.5 Colorimetric Method

Other method which becomes popular is calorimetric method. This method needs several days for growth of the bacteria but does not require radioactive substrate as the BACTEC (Newton et al., 2000). In addition, colorometric method is easy to perform, rapid, and inexpensive (Caviedes et al., 2002; Danizot & Lang, 1986; Mosmann, 1983). The advantage of this method is the visual observation can be affected by natural dark color of sample test. It is lead to the results are difficult to interpret and could lead to error (Primm & Franzblau, 2007). Colorimetric methods for detecting drug susceptibility of *M. tuberculosis* are generally based on the reduction of a reduction-oxidation (redox) indicator added to a liquid culture medium. The susceptibility of drugs is detected by a change in colour of the oxidation-reduction indicator, which is directly proportional to the number of viable mycobacteria in the medium (Yajko et al., 1995). There are several redox indicators that can be used for colorimetric method such as resazurin, nitrate, and tetrazolium salt.

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is a blue dye. Presence of growth of mycobacterial cell is indicated by the colour change from blue to pink. For susceptibility testing of mycobacteria, MIC of anti-TB drug is defined as the lowest drug concentration that prevented the colour change of resazurin (Nateche et al., 2006).

Susceptibility testing of mycobacteria is also evaluated using nitrate as redox indicator. Living mycobacterial cell converts nitrate (NO_3^-) to nitrite (NO_2^-) that indicates pink – violet color when added with Griess reagent prepared by mixing concentrated hydrochloric acid, sulphanilamide, and *n*-1-naphthylethylenediamine dihydrochloride solution. Antimycobacterial activity of a drug is interpreted if there is no color change (Coban et al., 2004).

Tetrazolium salt is used as the indicator since bacteria or fungi convert them to colored formazan derivatives that can be observed by colour change from yellow to purple (Grare et al., 2008). In order to implement a rapid and inexpensive method for determining the MICs of several anti-TB drugs, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay has also been formatted as a microplate-based test and evaluated with clinical isolates of *M. tuberculosis* (Morcillo et al., 2004).

MTT has been used widely for measuring cell proliferation and viability. Basically, the MTT is a yellow compound that changes to purple when reduced by dehydrogenase of active cells (Palomino et al., 2007). MTT is transported into the cell through endocytosis mechanism. Nicotinamide adenine dinucleotide plus hydrogen (NADH) and nicotinamide adenine dinucleotide phosphate plus hydrogen (NADPH), two enzymes continuously available in the living cell during cellular metabolism reduce the MTT to MTT formazan (Metzler, 2002). Furthermore, the

MTT formazan accumulated in the endosome compartment is transported to the cell surface and produces purple color when react to tetrazolium (Liu et al., 1997). Reduction of MTT to MTT formazan is described in Figure 1.4.

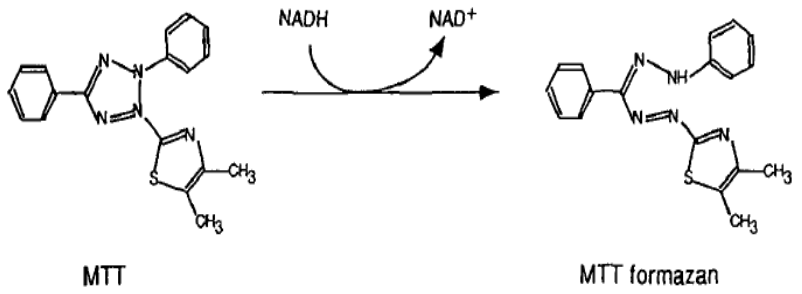

Figure 1.5 Reduction of MTT to MTT formazan

1.3 Uses of Traditional Medicine for Treatment of Tuberculosis

According to WHO (2008b), traditional medicine was defined as the sum total of knowledge, skills and practices based on the theories, beliefs, and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, improve or treat physical and mental illness. Medicinal plant and bioactive compounds have been used widely in the world. Approximately 80% of world's population still rely on medical plants for their primary healthcare (WHO, 2001). In Malaysia, Arabic Unani medicine and galenic philosophy are fundamental of Malaysian traditional medicine. It is rapidly progressed by influencing of other practices of Chinese, Indian, Indonesian and indigenous poeple traditional medicine. Chants, prayers, massage, abstinence and natural resources derived from plants are commonly used for cure several health problems (Jamal, 2006). More than 10,000 traditional medicine practitioners were currently reported by the government of Malaysia. Approximately, seventy percent of Malaysian population used traditional medicine for treat their health problems. However, there are a few of traditional

medicine types which are integrated to public hospital practices such as massage, acupuncture and herbal oncology (Abuduli et al., 2011).

Traditional medicines are used to cure tuberculosis in many countries. For example, in Indonesia, *Artocarpus elasticus* Reinw (Moraceae) is used to treat tuberculosis. The mature leaves are dried, crushed and then mixed with spices. The mixture is then cooked with water to obtain a decoction (Setyawati, 2009). In addition, *Pleomele angustifilia* (Agavaceae) has also been used for TB treatment. The leaf of this plant is boiled with water and then the water is taken (Roosita et al., 2008).

In addition, Mohamad et al. (2011) reported that *Flemingia strobilifera* (Fabaceae), *Licuala spinosa* Thunb. (Arecaceae), *Tabernaemontana coronaria* (Apocynaceae), and *Tenospera crispa* (Menispermaceae) have been used in the Malaysian community for tuberculosis treatment. According to Mat-Saleh and Latif (2002), the part used from these plants is commonly the leaf or stem. The people prepare the concoction by boiling the plant with water or soaking in water overnight to get a decoction.

McGaw et al. (2008) reported a large number of plants in South Africa that are used as traditional medicine for the treatment of tuberculosis. They include the family of Aizoaceae, Alliaceae, Asclepiadaceae, Asparagaceae, Asteraceae, Combretaceae, Euphorbiaceae, Fabaceae, Geraniaceae, Moraceae, Myrtaceae, Polygonaceae, Rubiaceae, Santalaceae, and Sapindaceae. The plants are commonly employed in aqueous infusions and then administered orally.

In Uganda, about 88 plants used for tuberculosis were identified. The most frequently used are *Eucalyptus* spp., *Ocimum suave* Willd., *Persea americana* Mill., *Acacia hockii* De Wild., *Warburgia salutaris* (G. Bertol.) Chiov., *Zanthoxylum*

chalybeum Engl., and *Momordica foetida* Schum. The people usually prepare their concoctions by decoction or infusion. Materials such as honey, rock salt, sugar, and *Zingiber officinale* Rosc. are also added in the concoction as preservatives. It is taken 3 times per day with dose 1 – 2 teaspoons for 1 – 12 weeks or until the patient recovers (Tabuti et al., 2010).

1.4 Plants as Antimycobacterial Agents Resources

The literature on the antibacterial, antituberculosis, antifungal, and antiviral properties of plant extracts has been progressed over the past decade (Newton et al., 2000). Interestingly, some of the plants used for TB in traditional medicine proven to have antimycobacterial activity. For example, about 149 species of Indian medicinal plants showed close correlation with ethnomedicinal uses for TB or related diseases (Gautam et al., 2007).

In Malaysia, 38 plants have been identified to have antimycobacterial activity with MICs ranging from 1600 – 400 µg/ml. The highest activity was demonstrated by *Angiopteris evecta* (J.R. Forst.) Hoffm. (Marattiaceae) with MIC value of 400 µg/ml (Mohamad et al., 2011).

McGaw et al. (2008) reported about 74 extracts derived from South African plants demonstrated antimycobacterial activity. The most active of the plant extracts has MIC value of 100 µg/ml, such as *Helichrysum caespitatum*, *Nidorella anomala* Steetz., *Chenopodium ambrosioides* Linn., *Euclea natalensis* A. de Candolle., *Croton pseudopulchellus* Pax., *Ekebergia capensis* Sparrm., *Salvia radula*, and *Polygala myrtifolia* Linn. In addition, Lall and Meyer (1999) also reported fourteen acetone extracts of South African medicinal plants showed inhibitory activity against mycobacterial. Some of them including *Chenopodium ambrosioides*, *Ekebergia*

capensis, *Euclea natalensis*, *Helichrysum melanacme*, *Nidorella anomala*, and *Polygala myrtifolia* were also shown to have activity against the resistant strain of *M. tuberculosis* with the MIC values ranging from 100 – 1000 µg/ml.

Jimenez-Arellanes et al. (2003) also reported 22 plants derived from Mexican medicinal plants indicated to have antimycobacterial activity with MIC value in the range of 50 – 200 µg/ml. Interestingly, among the plants, the hexane extract of *Lantana hispida* demonstrated potential activity when tested against the resistant strain of *M. tuberculosis* with the MIC values ranging from 25 – 100 µg/ml.

Meanwhile, Duke (1992) described more than 230 plants from various families that were used to treat tuberculosis. Some of them belong to Fabaceae, Asteraceae, Liliaceae, Acanthaceae, Piperaceae, Minispermaceae, and Araceae. For example, *Andrographis paniculata*, *Allium sativum, Artemisia annua*, *Aloe vera*, *Morinda citrifolia*, and several *Piper* species.

According to Gautam et al. (2007), in India, there were 25 most active plant species against mycobacteria such as *Acorus calamus*, *Adhatoda vasica*, *Allium sativum*, *Alpinia galanga*, *Artocarpus lakoocha*, *Caesalpinia pulcherrima*, *Calotropis gigantea*, *Canscora decussata*, *Cinnamomum camphora*, *Cissampelos pareira*, *Citrullus colocynthis*, *Erythrina variegata*, *Glycyrrhiza glabra*, *Inula racemosa*, *Juniperus excelsa*, *Morinda citrifolia*, *Ocimum sanctum*, *Piper cubeba*, *Plantago major*, *Portulaca oleracea*, *Psoralea corylifoila*, *Sassurea lappa*, *Solanum dulcamara*, *Tinospora cordifolia*, and *Zingiber officinale*. These plant species showed antimycobacterial activity with MIC values ranging from 10 to 100 µg/ml and the active compounds from 11 of these species have been isolated with MIC values ranging from 1 to 50 µg/ml.

Potential activity of naturally occurring compounds and synthetic analogues as well as extracts from plants and microorganisms against *M. tuberculosis* have also been reported by Okunade et al. (2004). The activity was shown in the MIC range of 1.56 – 100 µg/ml. Several phytochemicals which have potential antimycobacterial agents were also reported including ergosterol-5,8-endoperoxide from *Arjuga remota*, with MIC value of 1 µg/ml (Cantrell et al., 1999), a jujubogenin saponin from *Colubrina retusa* with the MIC of 10 µg/ml (ElSohly et al., 1999), and phenylethanoids from *Buddleja cordata* with the MIC of 64 µg/ml (Avecedo et al., 2000).

Compounds derived from plants that have potential as antitubercular agents include berberine, lichoisoflavone, erygibisoflavone, phaseollidin, erythrabyssin II, and tryptanthrin with MIC values in the range of 1 – 25 µg/ml (Mitscher & Baker, 1998). Two of the tannin groups identified as ellagitannin and punicalagin isolated from *Combretum molle* exhibited antimycobacterial activity with the MIC ranging from 600 – 1,200 µg/ml (Asres et al., 2001). Essential oils of *Achyrocline alata* and *Swinglea glutinosa* also showed activity against *M. tuberculosis* with MIC values of 62.5 µg/ml and 100 µg/ml, respectively (Bueno-Sanchez et al., 2009).

A drug is considered ideally effective if it has low adverse-effects, has good pharmacokinetic properties and cheap. Therefore, many studies have been made to discover antimycobacterial drugs from natural products. There is an increasing interest in natural products as potential therapeutic agents, including plant extracts. Developing new drugs based on natural product derived from plant indicates good prospect. This is triggered by the emergence of techniques to isolate and optimize the structure of lead compounds (Bisth et al., 2006).

Plants are incredible sources of various types of molecules that can be used as medicine, including the treatment of tuberculosis. A number of extracts and pure compounds derived from plant indicated potential activity against *Mycobacterium*. The metabolites such as alkaloids, phenolics, steroids, and terpenoids are known to have activity against mycobacteria. These phytochemicals provide useful prospect for the development of new antitubercular drugs which may act on newer target. It also will expand opportunities to discover the new generation of antitubercular agents (Negi et al., 2010).

Plants have been used worldwide in traditional medicines for the treatment of different diseases. The large diversity of plants in the world provide a basis of selecting the starting point for new drug discovery, especially novel lead compounds. The discoveries including searching for new anti-TB agents can be made by evaluating anti-TB of the plants based on the traditional use (Kobarfard, 2004).

One of the important groups of plant which have potential as antimycobacterial agent sources is family Piperaceae. Interestingly, several species of this family have shown activity against *M. tuberculosis* with the MIC values $\leq$ 100 µg/ml. As an extension of the above finding, in this study, we have focused on the antimycobacterial activity of *Piper* species at the cellular level to know its mechanism of action. In addition, we have also used the approach of metabolomics to obtain a picture of the plant metabolite responsible for the activity. To the best of our knowledge, this is the first study ever conducted in Malaysia.

1.4.1 Family Piperaceae

The family Piperaceae consists of 10 genera and about 2,000 species of tropical plant of which about 30 species are used in health system in Asia-Pacific

region (Wiart, 2006). These plants are widely distributed in tropical and subtropical regions of the world mainly in Asian tropics, African tropics, South Pacific, Central America, Atlantic forest, Choco region, Andes region, and Amazonia. The greatest diversity occurs in American tropics and Southern Asia where the economically important species *Piper nigrum* Linn. and *Piper betle* Linn. originated (Jaramillo & Manos, 2001). The genus *Piper* is usually used as food flavouring agents as well as in traditional medicines, and as pest control agents (Parmar et al., 1997).

Piper species are commonly grown as herbs, woody vines, and small trees. The leaves have a pungent flavor and grow singly. The plant also has numerous flowers which lack petals. The stems are usually creeping and climbing (Chaveerach et al., 2006). Most species of *Piper* are aromatic because of the essential oil cells in their tissues (Greig, 2004).

The family Piperaceae has widely been used in traditional medicinal system in many countries. The use of the family Piperaceae by commmunities are commmonly to treat gastric ulcers (Braga et al., 2007; Hammond et al., 1998), wound healing (Schmidt et al., 2009), malaria (Braga et al., 2007), rheumatism (Sanz-Biset et al., 2009; Zheng & Xing, 2009), cough (Green et al., 2010; Roosita et al., 2008; Hilgert, 2001), fever (Ong & Nordiana, 1999), central nervous system disorder (Tene et al., 2007), asthma (Savithramma et al., 2007; Ong & Nordiana, 1999), leprosy (Green et al., 2010), and tuberculosis (Gale et al., 2007; Billo et al., 2005).

Piperaceae species have also been investigated by many researchers for a number of pharmacological activities such as antioxidant (Yamaguchi et al, 2006; Dasgupta & De, 2004), antialergic (Wirotesangthong et al., 2008), antidiabetic (Arambewela et al., 2005), antileishmanial (Rocha et al., 2005), insecticidal agent